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Laboratory Investigation

Studies on the Structure and Chemistry of Dentin Collagen-Phosphophoryn Covalent Complexes

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Summary. Bovine and rat dentin contain aspartyl-phosphoseryl-enriched collagen-associated phosphoproteins which represent 1–2% of the mineral-free dry weight. These phosphophoryn moieties are not extracted by saturated neutral EDTA, pH 7.4, nor by guanidine hydrochloride-EDTA, pH 7.4. Cyanogen bromide degradation of the dentin matrix does release a high molecular weight fragment containing hydroxyprolyl, hydroxylysyl, prolyl, and glycyl residues as well as high concentrations of aspartyl and phosphoseryl residues, the amounts of which indicate a 50% collagen–50% phosphophoryn nature. Gel filtration and ion exchange chromatography under dissociative, denaturing conditions, as well as in the presence of disulfide bond reducing reagents failed to separate the collagen and phosphophoryn moieties. Hydroxyapatite, which selectively absorbs phosphophoryn, also failed to separate the collagenous component, leading to the conclusion that the moieties represented a covalent conjugate. ^{31}P NMR spectroscopy showed the bovine collagen-phosphophoryn complex to contain only phosphomonoesters similar to soluble phosphophoryn. Reduction with [^{3}H]$NaBH_4$, followed by cross-link analysis, did not reveal any reduced aldimine cross-link amino acids. Of the 4 hydroxylysyl residues/1000 in the intact bovine collagen-phosphophoryn complex, one-fourth are periodate resistant, indicating either O-or N-substitution. The periodate-resistant hydroxylysyl residues are located in bacterial collagenase-sensitive regions, and it is likely that these represent hydroxylysine O-glycosides. These data suggest that: (a) the collagenous component of the conjugate derives from a glycosylated peptide, probably α2CB4, and (b) the association is covalent, but does not involve disulfides, phosphate-, hydroxylysine-, or reducible aldehyde-mediated covalent bonds.

Key words: Phosphophoryn — Collagen — Dentin cross-linking — Mineralization.

Introduction

Dentin from all species examined to date contains phosphoproteins of unique amino acid composition as major components of the organic matrix [1]. These phosphoproteins, collectively called "phosphophoryns" because of their exceedingly high content of organic phosphorus [2], are present both as readily extractable [3, 4] and as insoluble moieties [5–7]. Our physical chemical studies have demonstrated that soluble phosphophoryn binds calcium ions at a number of high-affinity sites equivalent to the sum of phosphate and carboxylate groups per molecule, and that the protein assumes an ordered conformation and becomes insoluble as the calcium complex is formed [8]. In vitro phosphophoryn at high concentration can inhibit calcium phosphate precipitation from supersaturated solutions [9] while at low concentration it can accelerate the nucleating of hydroxyapatite [10].

In contrast to soluble phosphophoryns, the insoluble or nonextractable phosphophoryns are always firmly associated with the demineralized dentin collagen matrix [5–7], and in two recent studies [2, 11] evidence was presented which suggests a possible covalent association with collagen in both rat incisor and bovine molar dentin.

Emerging from our studies on both types of phosphophoryn in rat incisor and bovine molar dentin is our hypothesis that matrix-bound phosphophoryn might serve as the epitaxic agent in dentin mineral-

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ization [12, 13]. In this context we have, in the work discussed here, undertaken an examination of the detailed structure and chemistry of the covalent phosphophoryn-collagen complex in dentin.

Materials and Methods

EDTA Demineralization

The EDTA-soluble components of unerupted bovine molars and of rat incisors were extracted as described previously [8]. The insoluble residue remaining after exhaustive demineralization, referred to as dentin, was washed free of EDTA with many changes of distilled water, 4°C, for a week and then lyophilized. The guanidine hydrochloride (Gdn·HCl) soluble components of demineralized dentin were extracted by stirring in 6M Gdn·HCl, 0.05M Tris-HCl, 0.1M EDTA, pH 7.4, at 4°C for a week. The suspension was centrifuged in a Sorvall RC-2B centrifuge, using a SS-34 rotor, at 10,000 rpm for 30 min at 4°C. The guanidine-soluble extract in the supernate was dialyzed against water and lyophilized. The pellet was dialyzed against large volumes of cold distilled water for a week and then lyophilized.

Cyanogen Bromide Digestion

The procedure of Scott and Veis [14] was used for cyanogen bromide (CNBr) digestion of demineralized dentin. The final lyophilized CNBr digest was suspended in 0.1N acetic acid at 37°C, and then centrifuged at 10,000 rpm for 30 min in a Sorvall RC-2B centrifuge with the SS-34 rotor. The supernatant, predominantly the CNBr peptides of dentin collagen, was decanted. The pellet was resuspended in 100 ml of 0.1N acetic acid, 37°C, and the centrifugation repeated. The second pellet was removed and redigested with CNBr–70% formic acid and the acetic acid fractionation repeated. The final acetic acid insoluble fraction of dentin CNBr digests contained 4–5% of the starting demineralized dentin in both the bovine molar and rat incisor preparations.

To determine the effect of CNBr in 70% formic acid on phosphophoryns, especially their phosphoamino acid content, the following control experiments were performed. Phosphoserine, phosvitin, and the soluble phosphophoryn from molar dentin were digested with CNBr as described above, and after lyophilization and resuspension in buffer (0.1M ammonium bicarbonate, pH 7.8) the digested samples were tested for organic phosphorus and inorganic phosphorus contents. In each case, the organic phosphorus content was unchanged and the inorganic phosphorus content remained undetectable. These data establish that our conditions for CNBr digestion do not hydrolyze phosphoamino acids. Evidence that CNBr digestion does not cause phosphorus migration or internal rearrangements was provided by amino acid analysis before and after CNBr digestion. In all three cases the measured phosphoserine contents were unaffected by CNBr digestion.

DEAE-Cellulose Chromatography

The final acetic acid insoluble residue was dissolved in 0.05M Tris-HCl, pH 8.2, 6M urea, at 40°C, and centrifuged to remove approximately 20–30% insoluble material. The supernatant was applied to a DEAE-cellulose column equilibrated at 40°C in the same buffer. A linear gradient of NaCl (0.0–0.5M) in the same 6M urea-Tris buffer was applied. Eluted protein peaks, located by measuring absorbance at 230 nm, were dialyzed against water and lyophilized.

Hydroxyapatite Chromatography

The procedure of Bernardi and Kawasaki [15] was used for hydroxyapatite chromatography. A water-jacketed column (0.9 × 20 cm) was packed with Bio Gel HT (Bio Rad Labs) and equilibrated with 1mM potassium phosphate, pH 6.8, at 40°C. Samples dissolved in the same buffer were applied to the column and eluted by a linear gradient, 1mM to 1.5M potassium phosphate, pH 6.8. Conductivity measurements were used to monitor the gradient, and absorbance at 230 nm was used to locate protein peaks.

Sephacryl-6M Guanidine HCl Chromatography

A column (1.5 × 100 cm) of Sephacryl S-200 Superfine (Pharmacia) was packed at room temperature using 6M Gdn·HCl (Schwartz-Mann Ultrapure) in 0.02M Tris-HCl, pH 7.4 as eluant. Blue Dextran (Pharmacia), α_1(I), β_{11} and higher aggregates of acetic acid soluble skin collagen, and phosvitin (Nutritional Biochemicals Company) were used as molecular weight markers.

^{31}P Nuclear Magnetic Resonance Spectroscopy

The facilities and experimental protocol were identical to those described previously for bovine phosphophoryn [8].

^{3}H-Borohydride Reduction and Analysis of Reduced Cross-Links in Bovine Collagen-Phosphoprotein Complexes

Four milligrams of hydroxyapatite-derived collagen-phosphophoryn conjugate were reacted with ^{3}H-$NaBH_4$ (New England Nuclear Corporation) for 1 h in 0.1M sodium phosphate, pH 7.4 [16]. Reduction was terminated by addition of glacial acetic acid to pH 3.0 and the acidified solution was dialyzed against distilled water (Spectrapor-3, Spectrum Medical Industries) and lyophilized. One milligram of reduced conjugate was hydrolyzed in vacuo under N_2 using 2 ml 3N HCl, 105°C, 48 h. After evaporation to remove HCl, the hydrolyzate was dissolved in 2 ml of 0.2M Na citrate, pH 2.8. A 100 μl aliquot contained 1000 (±100) cpm; the specific activity of borohydride-reduced collagen phosphophoryn conjugate was 20,000 cpm/mg protein. The remaining 1.9 ml of hydrolysate was fractionated as described by Eyre [17], using an Aminex A-6 column (Bio Rad). The entire column effluent was collected in 2.3 ml fractions and counted for ^{3}H (2.0 ml effluent fractions plus 12 ml Packard Insta-Gel). Authentic dihydroxylysinonorleucine and monohydroxylysinonorleucine eluted at 32 and 40 ml, respectively. Efficiency of this ^{3}H-borohydride reduction procedure was evaluated by reduction, under identical conditions, of 10 mg of whole undemineralized chicken bone power, leading to incorporation of 1×10^6 cpm in the final demineralized bone collagen.

Degradation Studies

Collagen Digestion. Purified clostridial collagenase was prepared by the chromatographic method of Peterkofsky and Diegelman [18]. The digestion mixture contained, per 1 ml: 120 μmol HEPES buffer, pH 7.2; 0.0312 mg N-ethylmaleimide; 0.00735 mg $CaCl_2$; 50 μg collagenase [19]; and 3 mg collagen-phosphophoryn conjugate. The final digestion mixture was incubated at 37°C for 8 h. Addition of dry guanidine hydrochloride to a final concentration of 6M terminated the digestion.

Proteinase K Digestion. Proteinase K (EM Laboratories) digestion was carried out for 3 h at 37°C, using 200 μg of enzyme and 4 mg collagen-phosphophoryn conjugate in 1 ml of 0.01M Tris-HCl, pH 7.5.

Periodate Oxidation of Unsubstituted Hydroxylysyl Residues. The conditions for mild periodate oxidation were modified from those originally described by Schlueter and Veis [20]. Fractions containing known amounts of hydroxylysyl residues (from amino acid analysis of standard acid hydrolysates) were dissolved in 0.02M ammonium propionate, pH 6.4, containing 0.025M $NaIO_4$ and were incubated at room temperature in the dark for 16 h. An identical aliquot of the fraction was incubated in buffer without periodate and served as a control. The reaction was terminated by adding an excess of ethylene glycol. The extent of oxidation was assessed by amino acid analysis of an acid hydrolysate of the oxidized material.

Control studies were carried out in which 50 nmol of free hydroxylysine were incubated in 1 ml of the buffer, with and without added sodium metaperiodate, and showed: (a) quantitative recovery of hydroxylysine in the absence of sodium metaperiodate; (b) no detectable hydroxylysine in the presence of sodium metaperiodate and recovery of roughly 18 nmol of glutamic acid.

Reduction and Aklylation. The procedure for reduction of disulfide bonds and alkylation of cysteinyl residues was that of Monson and Bornstein [21].

Analytical Procedures

Phosphorus Analysis. The procedure used to quantify organic phosphorus was described previously [8].

Amino Acid Analysis. Amino acid compositions were determined using a single column procedure on a JEOL 6 AH amino acid analyzer. One milligram samples of protein were hydrolyzed for 22 h in triply distilled 6N HCl in vacuo at 108°C. Since no corrections for phosphoserine or serine losses were used, the values reported in Tables 1–3 are the direct analysis values after 22 h of acid hydrolysis.

Table 1. Amino acid compositions of bovine and rat dentin preparations, extracts, and derivatives[a]

Amino acid	Bovine dentin			Rat dentin	
	1 Demin. dentin	2 Gdn·HCl extract	3 HAc-insoluble CNBr digest	4 Demin. dentin	5 Gdn·HCl extract
Hydroxyproline	106	65	76	92	56
Aspartic acid	52	80	120	50	75
Threonine	16.7	28	19.6	19.9	34
Serine[b]	36	51	91	42	51
Glutamic acid	71	89	62	78	99
Proline	115	107	92	115	101
Glycine	318	228	269	320	238
½ Cystine	—[c]	8.0	2.7	T[f]	3.0
Valine	16.7	29	14.2	23	37
Methionine	8.8	12.9	T	4.5	4.1
Isoleucine	8.6	16.3	9.1	10.0	24
Leucine	27	51	25	25	52
Tyrosine	4.1	15.1	6.1	3.7	19.8
Phenylalanine	13.2	24	13.1	10.8	11.6
Histidine	4.8	12.0	6.8	3.8	13.9
Hydroxylysine	10.5	6.1	8.0	16.8	14.0
Lysine	20	33	26	18.9	38
Arginine	49	43	41	55	48
Phosphoserine[c]	3.1	5.5	29.4	ND[g]	ND
Homoserine[d]	0	0	T	—	—
Glucosamine[b]	1	5.6	T	ND	ND
Galactosamine[b]	—	T	T	ND	ND

[a] Residues per 1000 amino acid residues
[b] Uncorrected for losses on hydrolysis
[c] Phosphoserine after 22 h acid hydrolysis
[d] Homoserine + homoserine lactone
[e] —, not detected
[f] T, trace
[g] ND, not determined

Table 2. Amino acid compositions of DEAE-cellulose and of hydroxyapatite fractions of bovine and rat dentin acid-insoluble CNBr digest[a]

Amino acid	Fig. 3A			Fig. 5A	Fig. 5B
	F_1	F_2	F_3	D	D
Hydroxyproline	78	61	26	43	38
Aspartic acid	121	168	226	226	169
Threonine	21	16.7	10.9	17.6	31
Serine[b]	102	145	205	201	184
Glutamic acid	63	55	53	46	90
Proline	87	79	58	56	64
Glycine	270	222	173	156	159
Alanine	93	78	57	59	67
½ Cystine	T[c]	2.8	T	2.6	7.5
Valine	9.9	6.8	6.2	7.7	15.9
Methionine	T	3.2	T	T	7.5
Isoleucine	7.8	7.0	4.0	6.1	9.2
Leucine	22	19.3	13.4	16	25
Tyrosine	5.5	4.7	3.4	3.7	13.9
Phenylalanine	12.9	10.4	8.7	8.7	13.2
Histidine	6.7	4.9	6.7	6.5	12.2
Hydroxylysine	6.1	5.6	3.5	3.5	3.5
Lysine	22	27	30	33	21
Arginine	42	35	26	25	29
Phosphoserine[c]	26	46	68	79	31.2
Homoserine[d]	2.0	1.3	1.7	2.8	3.8
Glucosamine[b]	0.5	0.5	T	0.8	6.8
Galactosamine[b]	T	T	T	T	T

[a] Residues per 1000 amino acid residues
[b] Uncorrected for losses on hydrolysis
[c] Phosphoserine after 22 h acid hydrolysis
[d] Homoserine + homoserine lactose
[e] T, trace

SDS-Polyacrylamide Gel Electrophoresis. The method of Laemmli and Favre [22], with 6M urea in both stacking and running gels, was used unsuccessfully to analyze collagen-phosphophoryn complexes, as staining methods for Coomassie Blue [23] and Stains-All (Bio-Rad; [24]) failed to reveal protein bands. The reason for such non-ideal behavior of the collagen-phosphophoryn on SDS-polyacrylamide gel electrophoresis is not apparent, but is not surprising inasmuch as soluble phosphophoryns show similar non-ideal behavior (Dimuzio and Veis, 1978, and S. Lee, unpublished observations). This behavior was, however, in marked contrast to the high resolution we observed on SDS-polyacrylamide gel electrophoresis of purified collagen CNBr peptides in our system.

Results

Isolation of the Intact Collagen-Bound Phosphophoryn Moiety

The EDTA-demineralized insoluble bovine and rat dentin matrices obtained after removal of the neutral-soluble components have compositions expected for type I collagens (Table 1, columns 1 and 4). Except for the presence of phosphoserine, the nature of the noncollagenous components is obscured by the high content of collagen. The matrices were treated according to the flow diagram in Figure 1. The left-hand branch of the diagram represents extraction under typical protein denaturing, aggregate-dissociative conditions. Lee et al. [8] showed that phosphophoryn aggregates were soluble in and disaggregated by 6.0M Gdn·HCl at 4°C. The Gdn·HCl extracts of demineralized dentin contained very little protein, 0.08% and 0.07% of the weight of the dry EDTA-demineralized organic matrices from bovine molar and rat incisor dentins, respectively. Gel filtration of the bovine extract on Sephacryl S-200 in 6M Gdn·HCl yielded two fractions (Fig. 2), both of which proved to be essentially collagenous in nature, and were similar in composition to the unfractionated extract (Table 1, column 2). These data demonstrate that the demineralized dentin matrix contained undetectable amounts of soluble phosphophoryn at the conclusion of the EDTA demineralization; moreover, it appears that no collagen-bound phosphophoryn was extracted in 6M Gdn·HCl-EDTA.

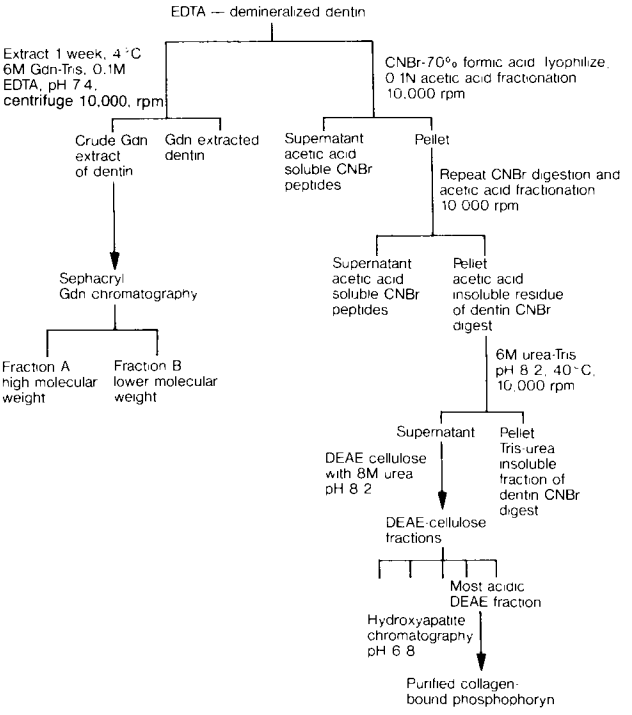

Fig. 1. Flow chart for isolation of collagen-associated phosphoprotein from dentin after EDTA-demineralization

Isolation of the Collagen-Phosphophoryn Conjugate via CNBr Digestion

Application of the procedures stipulated in the right-hand branch of Figure 1 similar to the proce-

Table 3. Effect of periodate and collagenase digestion on purified bovine collagen-phosphophoryn conjugate[a]

Amino acid	Sample treatment				
	Collagen-PP conjugate	After periodate[f] oxidation	After collagenase	After periodate[f] oxidation of collagenase-digested conjugate	Bovine soluble phosphophoryn
Hydroxyproline	65	64	10.0	7.8	0
Aspartic acid	115	163	305	232	376
Threonine	17.6	19.9	19.7	25	13.7
Serine[b]	124	169	312	296	340
Glutamic acid	58	60	47	81	37
Proline	81	71	25	23	T
Glycine	229	231	76	114	44
Alanine	78	81	28	49	13.2
½ cystine	11.1	7.8	8.2	0.5	3.6
Valine	12.8	16.1	18.9	16.5	5.5
Methionine[c]	3.6	—[g]	2.8	2.3	1.8
Isoleucine	7.5	10.5	6.0	8.7	3.5
Leucine	20	19.1	12.5	14.0	7.9
Tyrosine	5.1	—	6.5	6.3	4.1
Phenylalanine	10.2	0.4	6.3	7.4	4.9
Histidine	6.6	6.3	6.3	19.0	9.8
Hydroxylysine	4.3	1.7	0.5	T	0
Lysine	23	26	38	33	49
Arginine	35	34	10.3	9.6	5.6
Phosphoserine[d]	50	14.8	61	54	180
Homoserine[e]	2.1	2.9	—	—	8
Glucosamine[b]	T[h]	T	0.8	1.9	—
Galactosamine[b]	T	—	T	T	—

[a] Residues per 1000 amino acid residues
[b] Uncorrected for losses on hydrolysis
[c] Methionine + methionine sulfoxide
[d] Phosphoserine after 22 h acid hydrolysis
[e] Homoserine + homoserine lactone
[f] In addition to destruction of hydroxylysine several other amino acids are degraded. The chromatograms are complicated by the appearance of several additional, unidentified peaks. These were not included in the computation of composition.
[g] —, not detected
[h] T, trace amount

dure of Dickson et al. [7], led to the isolation of CNBr-cleaved matrix peptides. Unlike periodate solubilization of dentin, this method for CNBr digestion is a standard procedure, using dilute acid, and has never led to artificial cross-linking of proteins or glycoproteins. The acetic acid soluble CNBr peptides, although containing a small amount of phosphoserine, were essentially collagenous, in accord with previous experience [2, 7], and were not studied further. Since the first acetic acid insoluble fraction contained uncleaved methionyl residues, the CNBr digestion was repeated. The final CNBr-digested, acid-insoluble fraction represented 4–5% of the initial demineralized dentin and was considerably enriched in aspartic acid, serine, and phosphoserine (Table 1, column 3), all indicative of phosphophoryns. Since our control experiments (described in detail in Methods) showed that CNBr in 70% HCOOH did not cause phosphomonoester hydrolysis or phosphoryl rearrangements, this value, 4–5%, represents an upper limit for the collagen-phosphophoryn content of dentin.

Anion exchange chromatography on DEAE-cellulose by the procedure of Dickson et al. [7] produced similar chromatograms demonstrating a collagen-phosphophoryn conjugate, eluted by 0.3M NaCl. Since one of our goals was to determine the covalent or noncovalent nature of the conjugate, 6M urea was incorporated into the elution buffer at pH 8.2 and chromatography was carried out at 40°C. A small portion of the acid-insoluble fraction was insoluble in the chromatography buffer (6M urea, Tris·HCl, pH 8.2) and was removed by low-speed centrifugation. The bovine and rat prepara-

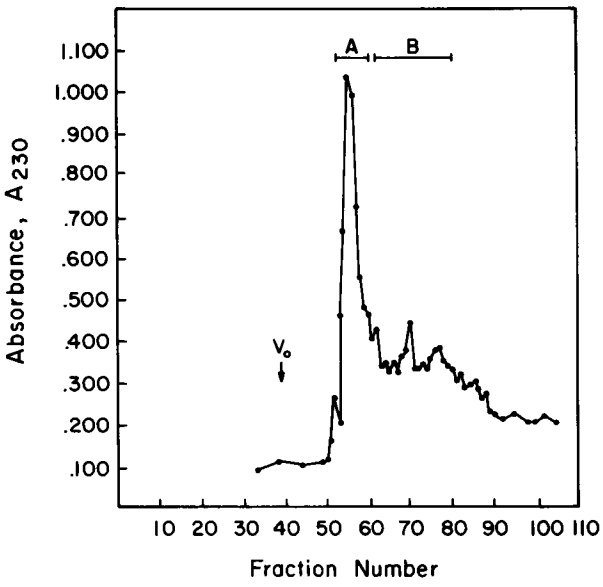

Fig. 2. Sephacryl S200, 6M guandine hydrochloride chromatography of the 6M guanidine hydrochloride extract of EDTA demineralized bovine dentin (before CNBr solubilization). V_0 indicates the elution of blue dextran. Soluble phosphophoryn elutes at fraction 57.

tions gave essentially identical chromatograms (Fig. 3) and differed from the chromatography in the absence of 6M urea only in shifting some protein from the high ionic strength eluting peak to one of a lower ionic strength. In the case of the bovine molar protein, every fraction contained collagen as indicated by the presence of hydroxylysine and hydroxyproline (data not shown). The pregradient peak (PG) of Figure 3A was entirely collagenous, the subsequent low ionic strength elution fractions were principally collagen, but also contained noncollagenous proteins. Peak F was not homogeneous as indicated by the variation in composition across the peak (Table 2, fractions F_1, F_2, and F_3). The most anionic material, eluted at the highest ionic strength, fraction F_3, was taken for further study as the collagen-phosphophoryn conjugate most likely to contain a covalent linkage region.

F_3 behaved as a single rather high molecular weight component when chromatographed on Sephacryl S-200 in 6M Gdn·HCl (Fig. 4). In contrast, soluble bovine phosphophoryn eluted behind

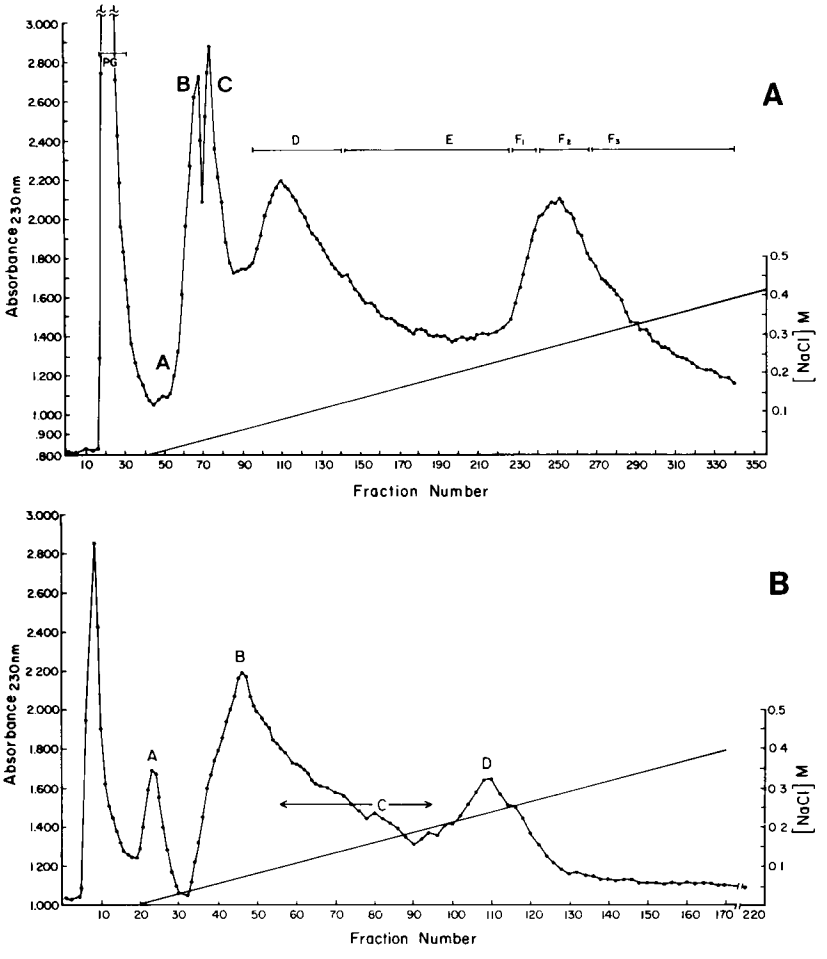

Fig. 3. A DEAE-cellulose chromatography of the acetic acid insoluble fraction of bovine dentin CNBr digest. Sample was applied on starting buffer (0.05M, pH 8.2, containing 6M urea) and was eluted by a linear NaCl gradient to 0.5M. Protein and NaCl elution were monitored by absorbance at 230 nm and conductivity, respectively. **3B** DEAE-cellulose chromatography of the acetic acid insoluble fraction of rat incisor dentin CNBr digest. All conditions and methods were identical to those in Fig. 3A.

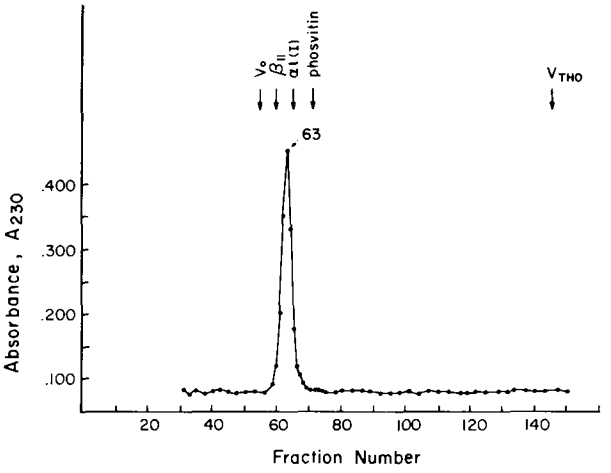

Fig. 4. Sephacryl S200, 6M guanidine hydrochloride chromatography of collagen-associated phosphoprotein from bovine dentin (i.e., the 0.3M NaCl-eluting fraction in Fig. 3A). Starting at the left of the chromatogram, arrows indicate elution volumes for: collagen γ-component, Blue Dextran, collagen β_{11} components, α1(I) chains, phosvitin, and ^3H_2O. Soluble bovine phosphophoryn had a larger V_e than phosvitin on this column

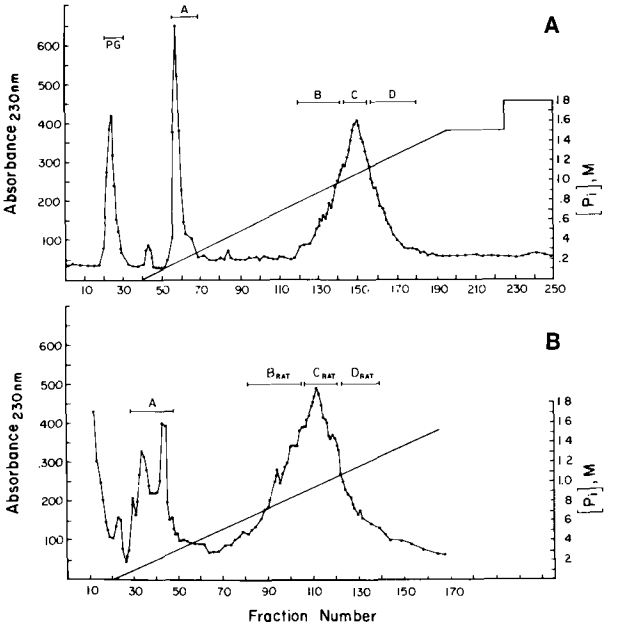

Fig. 5. A Hydroxyapatite chromatography of collagen-associated phosphoprotein from bovine dentin. The sample (fraction eluting at 0.3M NaCl in Fig. 3A) was applied in 1 mM NaP_i, pH 6.8, and eluted at the same pH by a linear NaP_i gradient to 1.5M. Protein and salt elution were monitored by absorbance at 230 nm and conductivity measurements respectively. **B** Hydroxyapatite chromatography of collagen-associated phosphoprotein from rat incisor dentin. The sample was the fraction eluting at 0.25–0.30M NaCl in Fig. 3B. All conditions were identical to those in Fig. 5A

phosvitin (fraction 73). Although the amino acid analysis (Table 2) indicated a trace amount of cysteine, the elution position of F_3 was unchanged after subjecting F_3 to reduction and alkylation of cysteinyl residues prior to chromatography. Taken together, Figures 3 and 4 show that binding between collagen and phosphophoryn in the complex does not involve disulfide bonding and that the linkage is stable in both 6M urea at 40°C and 6M Gdn·HCl at room temperature.

Denatured soluble type I collagen and its CNBr peptides are not adsorbed by hydroxyapatite in 1mM phosphate buffer [15], whereas soluble bovine phosphophoryn [10] and bovine collagen-phosphophoryn conjugate [11] are strongly adsorbed, requiring much higher phosphate concentrations for elution. The result of hydroxyapatite chromatography of F_3 is shown in Fig 5. The largest portion of F_3 requires phosphate concentrations of 1.0–1.2M for elution. Lesser amounts of other protein-collagen complexes are also obtained in pregradient or readily eluted components. The amino acid analyses (data not shown) show that these readily eluted components are distinctly different from the collagen-phosphophoryn complex. Fractions PG and A contain little phosphate, but do contain substantial quantities of amino sugars. PG and A both have high contents of glutamic acid and alanine as compared to the phosphophoryn complexes. On the other hand, each component has about the same relative content of hydroxylysine and hydroxyproline. PG and A contain only trace amounts of homoserine, whereas the phosphophoryn-containing components have between 2 and 3 residues of homoserine per thousand amino acid residues. Even at this stage the phosphophoryn-collagen complex peak is not homogeneous. Amino acid analysis of fractions B, C, and D showed the fractions to have steadily increasing contents of aspartic acid and phosphoserine as the phosphate concentration required for elution increases. Two explanations suggest themselves. The collagen-phosphophoryn complex might not be homogeneous, although the individual polypeptides in the mixture are tightly associated in a macromolecular complex that resists disruption by 6M urea or guanidine HCl. Alternatively, it is possible that the complex was homogeneous, but that it underwent limited proteolysis, either during or subsequent to hydroxyapatite chromatography, producing a mixture of smaller, similar complexes.

Gel filtration of bovine F_3 hydroxyapatite fractions B, C, and D on Sephacryl in 6M Gdn·HCl showed each fraction to contain a major component eluting at the same place, fractions 63–64. HA fractions B and C, however, both contained lower molecular weight components (Fig. 6). We have taken the major high molecular weight component as the collagen-phosphophoryn conjugate (Fig. 5A, Table

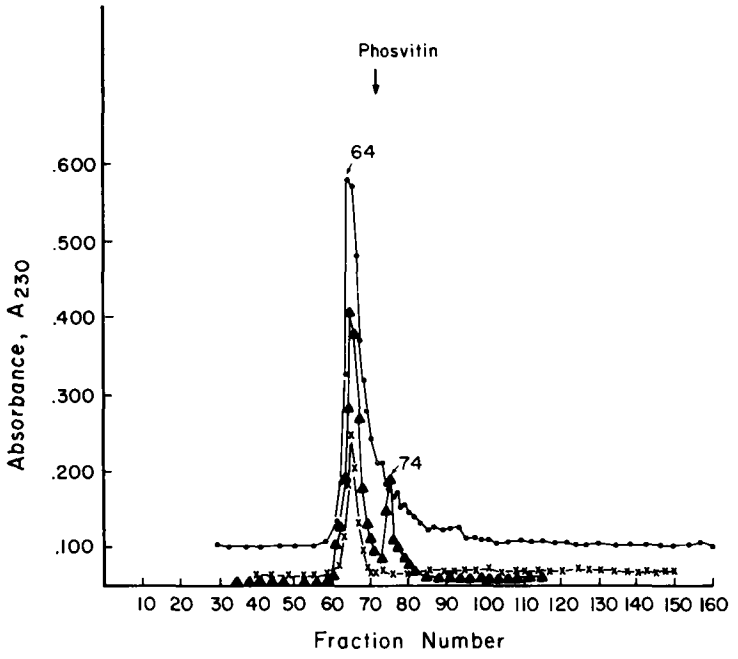

Fig. 6. Sephacryl S200, 6M guanidine hydrochloride chromatography of hydroxyapatite-purified collagen-associated phosphoprotein from bovine dentin. The following fractions from Fig. 5 were analyzed separately: ●●● fraction B from Fig. 5A; ▲▲▲ fraction C from Fig. 5A; and xxx fraction D from Fig. 5A.

2). Since we cannot dissociate the complex further in dissociative solvents, or on hydroxyapatite, we assume that it is covalent in nature.

When peak D of Figure 3B, the collagen-phosphophoryn conjugate fraction from rat incisors, was examined in similar fashion (Fig. 5B), the first major difference between the rat and bovine systems was evident. There was a much larger portion of a fraction eluting in the 0.6–0.8M phosphate range and this material was not phosphophoryn-like, since it was richer in glutamic acid than in aspartic acid or serine. The material eluting at $[P_i] > 0.9M$ was typically collagen-phosphophoryn (column 5, Table 2).

Studies on the Nature of the Linkage Region

Since treatment by procedures which would have reduced and alkylated any intermolecular disulfide bonds in the collagen-phosphophoryn conjugate did not separate the two moieties under dissociative conditions, the studies reported below focused on two possibilities: (a) the presence of a phosphorus-mediated bond; and (b) the presence of a lysine- or hydroxylysine-mediated bond.

Phosphorus-mediated Bonding. The possible involvement of orthophosphate esters in the linkage region of bovine dentin collagen-bound phosphoprotein was studied by ^{31}P nuclear magnetic resonance spectroscopy. The ^{31}P NMR spectrum of purified bovine collagen-bound phosphoprotein (after hydroxyapatite chromatography) in EDTA-D_2O (1:4, v/v, pH 9.5) is shown in Figure 7. There is a single phosphorus resonance at −4.1 ppm (150 Hz) downfield from the 85% H_3PO_4 standard. Resonances for free inorganic orthophosphate (−2.3 ppm), phosphodiesters (−0.5–2.0 ppm), and substituted pyrophosphates (10–12 ppm) are all absent [26, 27]. This chemical shift is consistent only with the presence of orthophosphate monoesters and is very similar to the chemical shift reported by Lee et al. [8] for bovine soluble phosphophoryn. Under identical conditions, bovine soluble phosphophoryn exhibited a single major resonance at −4.2 ppm (153 Hz) downfield from the H_3PO_4 standard. The possibility that 1% or less of the total organic phosphorus is present as phosphodiesters, phosphoramidates, or substituted pyrophosphates cannot be ruled out, since that amount would be below the limit of detection. Nevertheless, from these data we conclude that most, if not all, of the organic phosphorus in bovine collagen-bound phosphoprotein is present as orthophosphate monoesters and that the possibility of phosphorus-mediated cross-linkages is quite unlikely.

Lysine or Hydroxylsine Mediated Bonding: Absence of aldimine cross-link amino acids in collagen-phosphophoryn complex. Aldimines would result from allysine or hydroxyallysine reaction with amino groups of lysyl or hydroxylysyl residues [28]. After reduction with ^3H-borohydride, acid hydrolysis, and chromatography of the acid hydrolysate, every collagenous tissue exhibits a characteristic profile of ^3H-containing reduced cross-link amino acids [29, 30]. Since ^3H-dihydroxylysinonorleucine

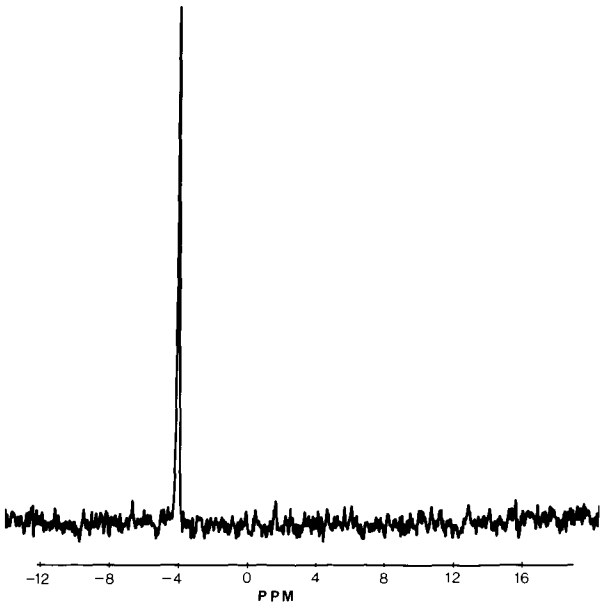

Fig. 7. ^{31}P NMR spectrum for collagen-associated phosphoprotein (fraction D, Fig. 5A) from bovine dentin, in D_2O-H_2O (1:3 v/v), apparent pH 9.4, 50 mM Na EDTA

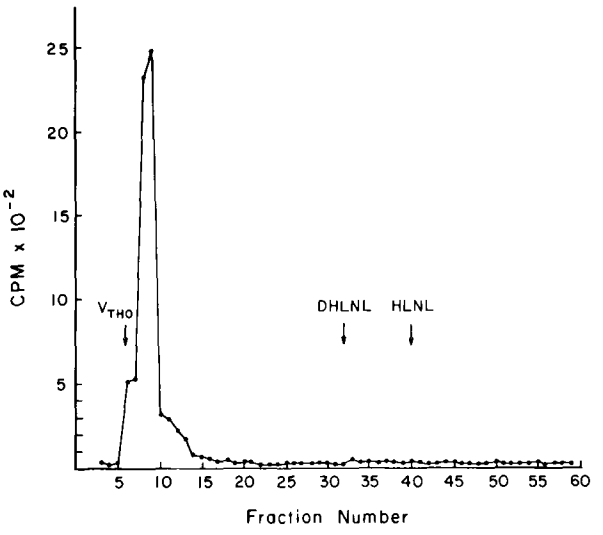

Fig. 8. Cross-link amino acid analysis of tritium-labeled amino acids from ^3H-$NaBH_4$-reduced collagen-associated phosphoprotein (bovine dentin). Elution volumes for tritiated water (THO), dihydroxylysinonorleucine (DHLNL), and hydroxylysinonorleucine (HLNL) are indicated by arrows. The rapidly eluting radioactivity (after THO) is considered unrelated to cross-link amino acids and their precursors

and ^3H-hydroxylysinonorleucine are the major and minor tritiated amino acids in acid hydrolysates of reduced bone and dentin collagens, the major and minor reducible cross-links are dehydrohydroxylysino hydroxynorleucine and dehydrohydroxylysinonorleucine, respectively [16, 30–33]. These cross-link components are not destroyed by CNBr digestion in 70% formic acid and have been recovered in their expected concentrations as ^3H-dihydroxylysinonorleucine and ^3H-hydroxylysinonorleucine among the soluble peptides even when CNBr digestion preceded borohydride reduction [16, 30, 33]. Therefore, it should be possible to detect any aldimine cross-linkages present in the CNBr-digested collagen-phosphophoryn conjugate peptide.

When the collagen-bound phosphophoryn from bovine dentin was analyzed for reducible cross-links, the results shown in Figure 8 were obtained. There was one peak of radioactivity emerging shortly after the void volume (V_{THO}) and there was no radioactivity in the elution positions for either dihydroxylysinonorleucine or hydroxylysinonorleucine (*arrows*). Since nearly all of the radioactivity eluted rapidly, far in advance of the known reduced cross-links and their precursors, and since this unidentified radioactive peak is typical in ^3H-$NaBH_4$-reduced collagens [31, 33], it is likely that this radioactive peak represents nonspecific reduction. Cysteic acid and phosphoserine which elute in this position may be the labeled components. Thus we conclude that the collagen-phosphophoryn linkage is not a reducible aldehyde-mediated cross-link.

The state of hydroxylysine. Since the collagen-phosphophoryn complex contains roughly 4 residues of hydroxylysine per 1000 residues, mild periodate oxidation was chosen to investigate the possible involvement of hydroxylysine in the putative linkage region. Unsubstituted hydroxylysyl residues would be oxidized by alkaline periodate and thus not appear in an amino acid analysis of an acid hydrolysate, whereas substituted hydroxylysyl residues (either N- or O-) would be periodate resistant and thus appear in an amino acid analysis of an acid hydrolysate. The data on the periodate oxidation of the bovine collagen-phosphophoryn conjugate are presented in Table 3. Of the total 4.3 residues per 1000 of hydroxylysine in the intact conjugate, roughly one-fourth are resistant to periodate oxidation and could represent either N-substituted hydroxylysine or O-substituted hydroxylysine, as in glycosides or esters.

In order to determine if the periodate-resistant hydroxylysine was involved in the linkage, as suggested by the earlier work of Carmichael et al. [6], we attempted to isolate the complex after degradation with collagenase, on the assumption that the phosphophoryn moiety could be eluted intact with only a small remaining portion of collagen that includes the linkage region.

The entire collagenase digestion mixture was passed over Sephacryl S200 in 6M Gdn·HCl (Fig. 9), and the high molecular weight component was

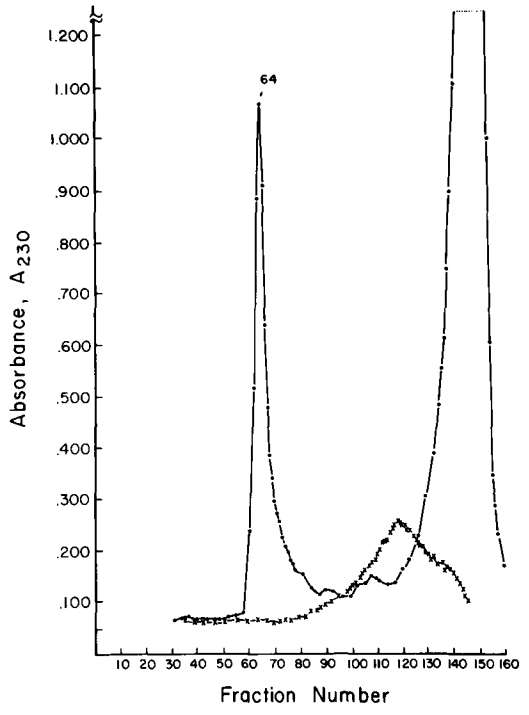

Fig. 9. Sephacryl S200, 6M guanidine hydrochloride chromatography of collagen-associated phosphoprotein (bovine) after in vitro proteolysis by purified bacterial collagenase (—•—•—•—) and by proteinase K (xxx). Chromatographic conditions were identical to those in Figs. 4 and 6

isolated. Amino acid analysis (Table 3) showed that the high molecular weight component was essentially phosphophoryn, with 305 and 312 residues per 1000 amino acid residues of aspartyl and seryl residues, respectively. Most of the hydroxyproline and glycine had been removed, but the fraction still contained 10 residues hydroxyproline per 100 amino acid residues and some hydroxylysine. Periodate digestion of this residual phosphophoryn conjugate fraction completely removed the remaining small amount of hydroxylysine (Table 3). These data, in conjunction with the observation that periodate degradation of the intact collagen-phosphophoryn conjugate demonstrated the presence of periodate-resistant hydroxylysine, showed that: (a) the periodate-resistant hydroxylysine residues in the intact conjugate occur in collagenase-sensitive regions and, in the above experiment, are removed in small peptides; and (1) the small amount of hydroxylysine left attached to the phosphophoryn after collagenase treatment is all periodate-sensitive and hence unsubstituted. Thus the hydroxylysine side chains cannot be involved directly in the covalent linkage between collagen and phosphophoryn. However, these data are equally clear in showing that a fragment of collagen containing hydroxyproline, and hence representing a helical region peptide sequence, is covalently bound to the phosphophoryn. Since the collagen CNBr peptide fragment had contained periodate-resistant, presumably O-glycosylated hydroxylysine residues, one or more of the glycosylated CNBr peptide sequence regions must be involved in the linkage.

We were surprised to find that the single high molecular weight component remaining after collagenase digestion eluted on Sephacryl S200 in the position of the undergraded collagen-phosphophoryn conjugate. Since calcium ions are required in the collagenase digestion and since these also cause phosphophoryn aggregation, EDTA was included in the chromatography buffer. The addition of EDTA did not change the elution position of the conjugate. Initially, we concluded that the conjugate had not been cleaved by collagenase, but this was disproved by the data of Table 3, indicating the removal of most of the collagenous components. These data could be interpreted as indicating that the collagen and phosphophoryn moieties fortuitously, in every system used, coelute, but this conclusion is not tenable because collagenase digestion leaves a collagen fragment of much different composition which still comigrates with the phosphophoryn. In fact, these data are strong evidence for the covalent nature of the collagen-phosphophoryn conjugate.

Digestion of the collagen-phosphophoryn conjugate with proteinase K, a relatively nonspecific protease, reduced the conjugate entirely to low molecular weight peptides (Fig. 9). Pronase, on the other hand, mimicks the effect of collagenase and degrades only the collagen portion of the moiety.

Discussion

The data presented above confirm earlier proposals [5–7, 11] that the collagen-phosphophoryn complex is a covalent conjugate of collagen and phosphophoryn. Carmichael et al. [6] had suggested that, since the conjugate contained a periodate-resistant hydroxylysine, the hydroxylysine might be involved in the cross-linkage. The present data clearly rule out the direct participation of hydroxylysine in the cross-linkage since the periodate-treated collagenase-digested phosphophoryn, which must still contain a collagen peptide fragment since it contains hydroxyproline, has no periodate-resistant hydroxylysine. Furthermore, the $[^3H]NaBH_4$ reduction gave no evidence for the presence of either allysine or hydroxyallysine or any of their reducible cross-links or cross-link precursors.

The ^{31}P NMR data likewise present no evidence for the presence of phosphate diester or pyrophos-

phate-mediated cross-linkages, although the possibility is not precluded with the certainty that hydroxylysine particpation can be ruled out. On the basis of the organic phosphorus content, and the ^{31}P NMR data indicating the exclusive presence of phosphomonoesters, we calculate that the collagen-phosphophoryn conjugate contained approximately 175 residues phosphoserine per 1000 amino acid residues. One or two diester phosphates per 1000 might escape detection.

The surprising behavior of the collagenase-digested conjugate on the Sephacryl S200 column in 6.0M Gdn·HCl provides the most revealing clue as to the nature of the conjugate. These data indicate that, in spite of the loss of about 50% of the peptide, the conformation and effective radius of gyration of the remaining phosphophoryn are essentially the same in 6.0M Gdn·HCl. Since in this solvent the most likely conformation is that of a random chain in both conjugate and collagenase-digested conjugate, the only tenable conclusion is that the two moieties are linked in a ladder-like fashion. That is, the collagen peptide chain and the phosphophoryn backbone must be stretched out alongside each other in the intact conjugate. For this to occur, one could imagine several binding sites. Since we have eliminated lysine aldehyde-derived and phosphate ester linkages, the strongest possibility would appear to be the presence of several ϵ-lysyl-γ-glutamyl bonds. Both the phosphophoryn and collagen moieties of the conjugate contain lysyl and glutamyl residues. The presence of ϵ-(γ-glutamyl) lysine isopeptides has been established in many intracellular and extracellular proteins. The bond is formed by the action of enzymes called transglutaminases [34, 35], and transglutaminase activity is abundant, at least in normal human fibroblasts [36]. Our current studies are aimed at the use of the proteinase K digests, followed by other proteases to reduce the conjugate to free amino acids plus the putative peptide cross-linkages.

From the structural point of view, the nature of the conjugate is as interesting as its chemistry. It would appear that if there are several covalent attachments, there should be several collagen peptide fragments on the conjugate. This implies, since we are dealing with a collagen helix region, that the phosphophoryn moiety might be stretched out along the helix backbone in the direction of the fiber axis. The generality of the collagen-phosphophoryn conjugate in dentin is shown by the similar behavior of the rat incisor and bovine molar systems. The fact that the conjugate, before collagenase digestion, does contain periodate-resistant hydroxylysine suggests that the collagen portion involved contains a glycoylated hydroxylysine. Taken together with the earlier data of Scott and Veis [25] demonstrating a decrease in the peptides α1CB6 and α2CB4 in the acid-soluble CNBr peptide digest fraction, it is likely that one or the other of these glycosylated collagen sequence regions [37] is the phosphophoryn binding region. The absence of reducible cross-linkages or cross-linkage precursors in the conjugate narrows possibilities further and implicates α2CB4 most strongly.

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